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Reactive Oxygen Species Contribute to Lipopolysaccharide-Induced Teratogenesis in Mice

Lei Zhao, Yuan-Hua Chen, Hua Wang, Yan-Li Ji, Huan Ning, Su-Fang Wang, Cheng Zhang, Jin-Wei Lu, Zi-Hao Duan, and De-Xiang Xu[1]

Department of Toxicology, Anhui Medical University, Hefei 230032, China

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Lipopolysaccharide (LPS) has been associated with adverse developmental outcome, including embryonic resorption, fetal death and growth retardation, and preterm delivery. In the present study, we showed that an ip injection with LPS daily from gestational day (gd) 8 to gd 12 resulted in the incidence of external malformations. The highest incidence of malformed fetuses was observed in fetuses from dams exposed to 20 μg/kg LPS, in which 34.9% of fetuses per litter were externally malformed. In addition, 17.4% of fetuses per litter in 30 μg/kg group and 12.5% of fetuses per litter in 10 μg/kg group were externally malformed. Importantly, external malformations were also observed in fetuses from dams exposed to only two doses of LPS (20 μg/kg, ip) on gd 8, in which 76.5% (13/17) of litters and 39.1% of fetuses per litter were affected. LPS-induced teratogenicity seemed to be associated with oxidative stress in fetal environment, measured by lipid peroxidation, nitrotyrosine residues, and glutathione (GSH) depletion in maternal liver, embryo, and placenta. alpha-Phenyl-N-*t*-butylnitrone (PBN, 100 mg/kg, ip), a free radical spin-trapping agent, abolished LPS-induced lipid peroxidation, nitrotyrosine residues, and GSH depletion. Consistent with its antioxidant effects, PBN decreased the incidence of external malformations. Taken together, these results suggest that reactive oxygen species might be, at least partially, involved in LPS-induced teratogenesis.

Key Words: **lipopolysaccharide; teratogenicity; reactive oxygen species.**

Lipopolysaccharide (LPS) is a toxic component of cell walls in gram-negative bacteria and is widely present in the digestive tracts of humans and animals (Jacob *et al*., 1997). Humans are constantly exposed to low levels of LPS through infection. Gastrointestinal inflammatory diseases and excess alcohol intake are known to increase permeability of LPS from gastrointestinal tract into blood (Zhou *et al*., 2003). High levels of LPS have also been detected in women with bacterial vaginosis (Platz-Christensen *et al*., 1993). In human, Gram-negative bacterial infections are a recognized cause of fetal loss and preterm labor (Romero *et al*., 1988). Mimicking maternal infection by exposing the pregnant rodents to LPS at early gestational stages resulted in embryonic resorption and fetal death (Gendron *et al*., 1990; Ogando *et al*., 2003). Maternal LPS exposure at middle gestational stages caused fetal death and preterm delivery (Leazer *et al*., 2002). We and others found that maternal LPS exposure at late gestational stages led to fetal death, growth restriction, skeletal development retardation, and preterm labor and delivery (Buhimschi *et al*., 2003; Rivera *et al*., 1998; Xu *et al*., 2005, 2006a, 2007).

Relatively few studies have investigated LPS-induced teratogenicity. Several earlier studies found that maternal LPS exposure resulted in the development of malformed fetuses in rats (Ornoy and Altshuler, 1976) and golden hamsters (Collins *et al*., 1994; Lanning *et al*., 1983). Recent studies showed that subcutaneous injection of LPS led to fetal malformation including exencephaly and eye deformities (Carey *et al*., 2003; Chua *et al*., 2006). However, the exact mechanism of LPS-induced teratogenesis remains unclear.

Several studies found that antioxidants, such as alpha-phenyl-N-t-butylnitrone (PBN), melatonin, N-acetylcysteine (NAC), and ascorbic acid, protected mice against LPS-induced fetal death, growth restriction, and preterm labor and delivery (Chen *et al*., 2006a, b; Xu *et al*., 2005, 2006b). On the other hand, excess reactive oxygen species (ROS) formation has been implicated in the teratologic mechanism of several chemicals, including phenytoin, benzo[*a*]pyrene, and thalidomide (Kasapinovic *et al*., 2004; Parman *et al*., 1999; Winn and Wells, 1997). The role of ROS in embryonic dysmorphogenesis in diabetic pregnancy has also been demonstrated (Cederberg *et al*., 2001; Sakamaki *et al*., 1999; Viana *et al*., 2000). A recent study showed that ascorbic acid inhibited ethanol-evoked ROS production and protected embryos of *Xenopus* against ethanol-induced growth retardation and microencephaly (Peng *et al*., 2005). The present study aimed to investigate whether maternal LPS exposure causes external and skeletal abnormalities, and to assess the potential role of ROS in LPS-induced teratogenesis.

[1] To whom correspondence should be addressed at Department of Toxicology, Anhui Medical University, Hefei 230032, China. Fax: +86-551-5161179. E-mail: xudex@mail.hf.ah.cn.

Lei Zhao, Yuan-Hua Chen, and Hua Wang contributed equally to this work.

MATERIALS AND METHODS

Chemicals. LPS (*Escherichia coli* LPS, serotype 0127:B8) and PBN were purchased from Sigma Chemical Co. (St Louis, MO). All the other reagents were from Sigma (St Louis, MO) or as indicated in the specified methods.

Animal husnandry and treatments. The CD-1 mice (8–10 weeks old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River (Beijing, China) whose foundation colonies were all introduced from Charles River Laboratories, Inc. (Wilmington, Massachusetts, USA). For mating purposes, four females were housed overnight with two males starting at 9:00 P.M. Females were checked by 7:00 A.M. the next morning, and the presence of a vaginal plug was designated as gestational day (gd) 0. Four pregnant mice were housed per cage. Food (Laboratory Rodent Chow No. 1) was provided by the Center for Laboratory Animal Sciences at Anhui Medical University (Hefei, China). The animals were allowed free access to food and water at all times and were maintained on a 12-h/12-h light/dark cycle in a controlled temperature (20–25°C) and humidity (50 ± 5%) environment. The present study consisted of two separate experiments.

Experiment 1. To investigate LPS-induced teratogenesis, the pregnant mice were divided into four groups randomly. In LPS-treated groups, the pregnant mice were ip injected with different doses of LPS (10, 20, or 30 μg/kg) daily (7:00 A.M.) from gd 8 to gd 12. The saline-treated pregnant mice served as controls. Maternal weights were recorded at 7:00 A.M. on gd 8, 12, 15, and 18. All animals were inspected daily for clinical signs and determined whether a pregnancy loss had occurred according to clinical signs and maternal weight. The remaining dams were sacrificed on gd 18 for fetal examinations.

Experiment 2. To investigate the protective effect of PBN on LPS-induced teratogenesis, all pregnant mice except controls were injected with two doses of LPS, one (20 μg/kg, ip) administered at 7:00 A.M. on gd 8 and the other (20 μg/kg, ip) administered at 3:00 P.M. on gd 8. Some pregnant mice were pretreated with PBN (100 mg/kg, ip) 30 min before both LPS treatments. Twelve dams each group were sacrificed 6 h after the second LPS treatment. Maternal liver, placenta, and embryo were excised for measurements of thiobarbituric acid–reactive substance (TBARS) and glutathione (GSH) content. Placentas were excised for measurement of nitrotyrosine residues. Maternal serum and amniotic fluid were collected for analysis of nitrite plus nitrate concentration. For remaining dams, maternal weights were recorded on gd 8, 12, 15, and 18. All animals were inspected daily for clinical signs and determined whether a pregnancy loss had occurred according to clinical signs and maternal weight. The remaining dams were sacrificed on gd 18 for fetal examinations.

All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Fetal examination. The dams were sacrificed on gd 18. The uterine horns were exposed and weighed. Live, dead, and resorbed fetuses were counted. Live fetuses were sexed, weighed, and examined for external morphological malformations. All live fetuses were stored in ethanol a minimum of 2 weeks. The fetuses stored in ethanol were cleared of skin, viscera, and adipose tissue. Fetuses were then incubated in acetone overnight and subsequently macerated and stained with alizarin red S for 2 days. After an overnight incubation in 70% ethanol/glycerol/benzyl alcohol, the fetuses were stored in glycerol until skeletal examination.

Tissue preparation and biochemical analysis. For the preparation of liver homogenates, 0.3 g of maternal liver was homogenized on ice in 3 ml of homogenization buffer (50 mM Tris–HCl, 180 mM KCl, 10 mM ethylenediaminetetracacetic acid, pH 7.4). For the preparation of placental homogenates, three placentas from same litter were homogenized on ice in 3 ml of homogenization buffer. A total of 36 placentas from 12 dams for each group were used for this analysis. For the preparation of fetal homogenates, six fetuses from same litter were pooled and homogenized on ice in 1 ml of homogenization buffer. A total of 72 fetuses from 12 dams for each group were used for this analysis. TBARS was determined in maternal liver and placenta according to Ohkawa *et al.* (1979). TBARS levels were expressed as nmol/mg protein. GSH in maternal liver, placenta, and fetuses was measured as described by Griffith (1980). GSH contents were expressed as nmol/mg protein. Protein contents were measured according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Analysis of nitrite plus nitrate. Nitrate plus nitrite, the stable end products of L-arginine–dependent nitric oxide synthesis, were measured in maternal serum and amniotic fluid using a colorimetric method based on the Griess reaction (Grisham *et al.*, 1996).

Immunohistochemistry. Three placental tissues per litter were fixed with 10% neutral formalin. Immunohistochemistry studies were performed to detect nitrotyrosine residues in mouse placenta. Briefly, placental tissue sections (5 μm) were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in absolute methanol for 15 min at room temperature. Then, sections were rehydrated through a graded series of ethanol and processed by using the streptavidin–peroxidase (SP) technique. Slides were heat-treated for antigen retrieval using citrate buffer (0.01 M, pH 6.0) in a microwave oven at maximal power four times for each 6 min. Sections were incubated in 5% normal goat serum for 30 min at room temperature, and then incubated nitrotyrosine rabbit polyclonal antibody (dilution 1:100) for 18 h at 4°C. Later, sections were incubated in goat anti-rabbit secondary antibody for 30 min at 37°C and SP complex for 30 min at 37°C. All antibodies were diluted in phosphate-buffer saline (PBS). Color development was performed with a solution containing 0.05% 3,3′-diaminobenzidine plus 0.01% hydrogen peroxide in PBS for 3–10 min. Control slides were performed by PBS instead of primary antibody. After color development, sections were counterstained with hematoxylin. Slides were dehydrated, cleared in xylene, mounted with DPX, and observed with a light microscope.

Statistical analysis. The litter was considered the unit for statistical comparison among different groups. Fetal malformation was calculated per litter and then averaged per group. For fetal weight and crown–rump length, the means were calculated per litter and then averaged per group. All quantified data were expressed as means ± SEM. at each point. $p < 0.05$ was considered statistically significant. Analysis of variance and the Student–Newmann–Keuls *post hoc* test were used to determine differences among different groups.

RESULTS

LPS-Induced Teratogenicity

In experiment 1, the pregnant mice were administered a 5-day (from gd 8 to gd 12) injection with LPS. No signs of maternal toxicity were observed in dams treated with LPS. Although LPS exposure deferred weight gain of the pregnant mice (Fig. 1A), no significant difference in maternal pure weights (maternal weights after fetuses were extracted) was observed among different groups (Fig. 1B)

Pregnancy Outcomes

A 5-day exposure resulted in pregnancy loss (Table 1). Only 9 out of 17 dams (52.9%) in 30 μg/kg group completed the pregnancy, whereas 85.7% (12/14) of dams in 10 μg/kg group and 84% (21/25) of dams in 20 μg/kg group completed the pregnancy. The number of litters, implants per litter, resorptions per litter, live fetuses per litter, and dead fetuses per litter is presented in Table 1. There were no differences in

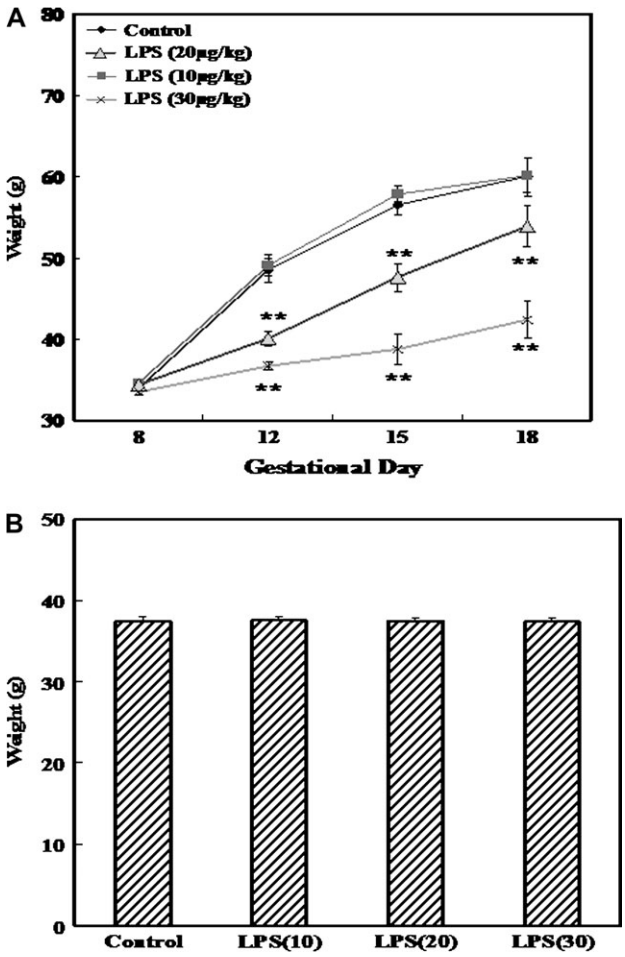

FIG. 1. Effects of a 5-day LPS exposure on maternal body weights. The pregnant mice were intraperitoneally injected with different doses of LPS (10, 20, or 30 μg/kg) daily from gd 8 to gd 12. (A) The pregnant body weights on gd 8, 12, 15, and 18. (B) The extrauterine body weights. Data were expressed as means ± SEM. $^{**}p < 0.01$, as compared with control group.

the number of implantation sites among different groups. A 5-day exposure did not increase embryonic resorptions. The effects of maternal LPS exposure on fetal death are presented in Figure 1B. A 5-day exposure significantly increased fetal mortality.

External Malformations

LPS-induced external malformations are presented in Figure 2. A 5-day exposure significantly increased the incidence of litters with malformed fetuses. 77.8% (7/9) of litters in 30 μg/kg group, 61.9% (13/21) of litters in 20 μg/kg group, and 41.7% (5/12) of litters in 20 μg/kg group were affected (Fig. 2A). The highest incidence of malformed fetuses was observed in fetuses from dams exposed to 20 μg/kg LPS, in which 34.9% of fetuses per litter were externally malformed. 17.4% of fetuses per litter in 30 μg/kg group and 12.5% of fetuses per litter in 10 μg/kg group were externally malformed. Exencephaly and encephalomeningocele were two of the most common malformations. Among dams exposed to 20 μg/kg LPS, 25.9% of fetuses per litter were either exencephaly or encephalomeningocele. In addition, 21% of fetuses per litter in 20 μg/kg group had eye deformities (Fig. 2B). Other external malformations observed included short tail, omphalocele, micromelia, and syndactyly.

Skeletal Abnormalities

The effects of maternal LPS exposure on skeletal development are presented in Fig. 2B. A 5-day exposure significantly increased the incidence of skeletal abnormalities. In dams exposed to 30 μg/kg LPS, more than 80% of fetuses per litter had skeletal abnormalities.

Fetal Weight and Crown–Rump Length

The effects of maternal LPS exposure on fetal weight and crown–rump length are presented in Table 2. A 5-day exposure significantly decreased crown–rump length. Correspondingly, a 5-day exposure significantly decreased fetal weight.

Role of ROS in LPS-Induced Teratogenesis

In experiment 2, the pregnant mice were injected with two doses LPS, one (20 μg/kg, ip) administered at 7:00 A.M. on gd 8 and the other (20 μg/kg, ip) administered at 3:00 P.M. on gd 8. No signs of maternal toxicity were observed in dams treated with LPS. However, the dams pretreated with PBN showed toxicity, manifested as pilorection, squinting, and decreased motor

TABLE 1
The Effects of a 5-Day LPS Exposure on Fetal Outcomes

Dose (μg/kg)	Litters	Pregnancy loss (%)	Successful pregnancy (*n*)	Implantation sites per litter (±SEM)	Resorptions per litter (±SEM)	Live fetuses per litter (±SEM)	Dead fetuses per litter (±SEM)
0	19	0	19	12.8 ± 0.48	0.6 ± 0.25	12.2 ± 0.48	0.1 ± 0.25
10	14	2 (14.3)	12	13.5 ± 1.01	0.8 ± 0.21	11.4 ± 1.41	1.3 ± 0.74
20	25	4 (16.0)	21	13.7 ± 0.61	1.0 ± 0.37	10.3 ± 0.94	2.7 ± 0.72**
30	17	8 (47.1)	9	13.4 ± 0.63	0.7 ± 0.67	9.3 ± 1.53	3.4 ± 1.07**

Note. $^{**}p < 0.01$ as compared with controls.

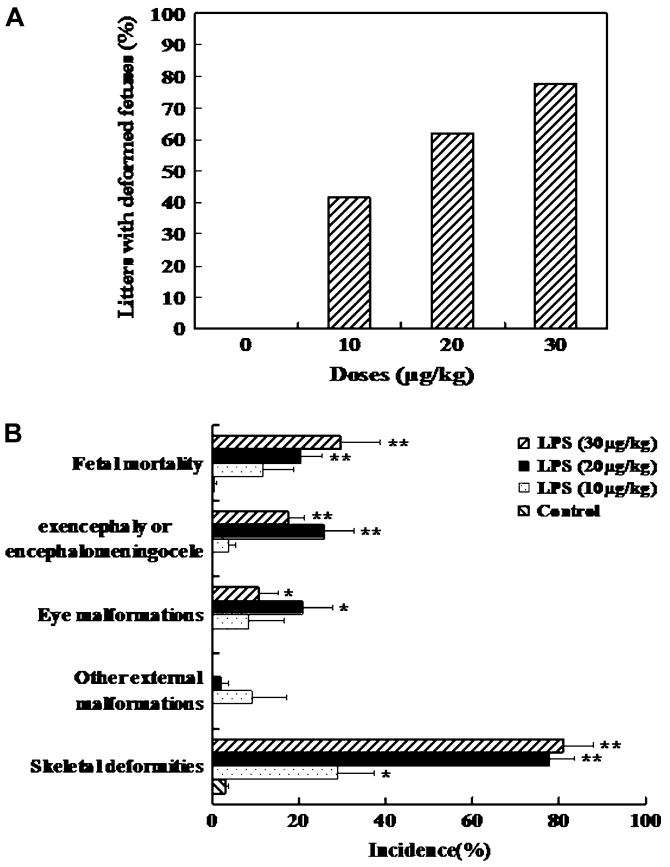

FIG. 2. LPS-induced external malformations. The pregnant mice were intraperitoneally injected with different doses of LPS (10, 20, or 30 μg/kg) daily from gd 8 to gd 12 and sacrificed on gd 18. The numbers of live fetuses and dead fetuses were counted. Live fetuses were examined for external malformations and skeletal abnormalities. (A) The percentage of litters with externally malformed fetuses in each group. (B) The incidence of fetuses with external malformations and skeletal abnormalities. Fetal malformations were calculated per litter and then averaged per group. Data were expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ as compared with control group.

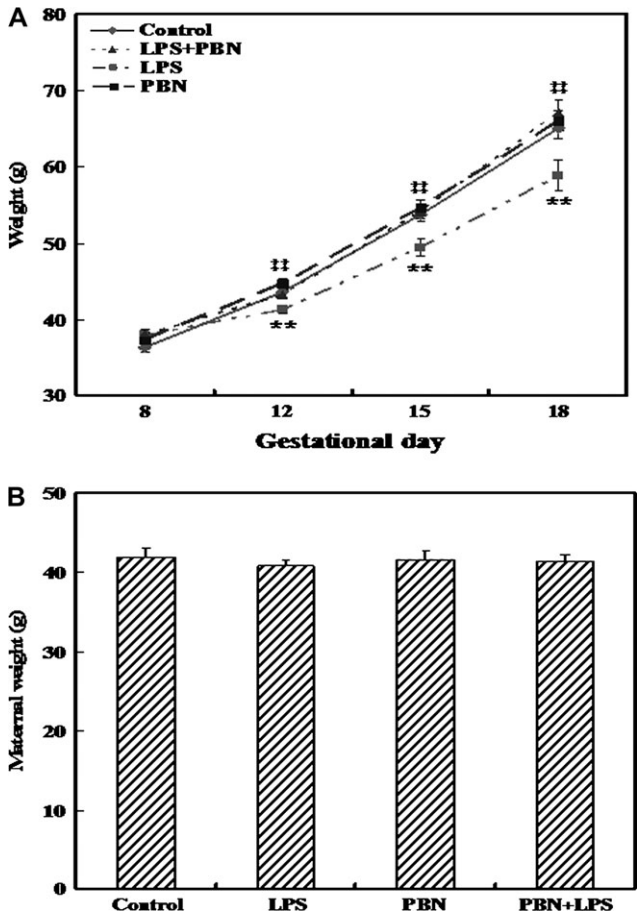

FIG. 3. Effects of a two-dose LPS exposure on maternal body weights. The pregnant mice were intraperitoneally injected with two doses of LPS, one (20 μg/kg) injected on gd 8 and the other (20 μg/kg) injected 8 h later. In PBN group, the pregnant mice were pretreated with PBN (100 mg/kg, ip) 30 min prior to LPS. (A) The pregnant body weights on gd 8, 12, 15, and 18. (B) The extrauterine body weights. Data were expressed as means ± SEM. $**p < 0.01$, as compared with control group. $‡‡p < 0.01$ as compared with group pretreated with PBN.

activity. Although LPS exposure deferred weight gain of the pregnant mice (Fig. 3A), no significant difference in maternal pure weights (maternal weights after fetuses were extracted) was observed among different groups (Fig. 3B).

TABLE 2
Effects of a 5-Day LPS Exposure on Fetal Weight and Crown–Rump Length

Dose (μg/kg)	Litters (*n*)	Fetal weight (g, ±SEM)	Crown–rump length (mm, ±SEM)
0	19	1.47 ± 0.03	23.3 ± 0.12
10	12	1.44 ± 0.08	23.3 ± 0.40
20	21	1.13 ± 0.05††	21.4 ± 0.44**
30	9	0.96 ± 0.06††	20.1 ± 0.47**

Note. $**p < 0.01$ as compared with controls.

Pregnant Outcomes

The number of litters, implants per litter, resorptions per litter, live fetuses per litter, and dead fetuses per litter is presented in Table 3. There were no differences in the number of implantation sites among different groups. LPS-induced fetal death is presented in Fig. 4. As expected, two doses LPS greatly increased fetal mortality (3.3 ± 1.3% vs. 32.8 ± 7.9%, $p < 0.01$), whereas there was no difference in embryonic resorptions between LPS-treated mice and controls (Table 3 and Fig. 4).

External Malformations

As shown in Figure 4, the incidence of external malformations was significantly increased in fetuses from dams exposed to LPS, in which 76.5% (13/17) of litters and 39.1% of fetuses per litter were affected. In addition, 76.6% of fetuses per litter

TABLE 3
The Protective Effects of PBN on Fetal Outcomes

Groups	Litters	Pregnancy loss (%)	Successful pregnancy (*n*)	Implantation sites per litter (±SEM)	Resorptions per litter (±SEM)	Live fetuses per litter (±SEM)	Dead fetuses per litter (±SEM)
Control	10	0 (0)	10	14.9 ± 0.39	0.4 ± 0.27	13.7 ± 0.54	0.0 ± 0.29
LPS	18	1 (5.6)	17	15.2 ± 3.60	0.9 ± 0.32	9.4 ± 1.31*	4.9 ± 1.21*
PBN	12	4 (0)	12	15.0 ± 0.61	0.6 ± 0.27	14.4 ± 0.94	0.0 ± 0.00
PBN + LPS	16	8 (0)	16	16.0 ± 3.40	0.4 ± 0.20	14.6 ± 0.83‡	1.0 ± 0.30‡

Note. $^{*}p < 0.05$ as compared with controls. ‡$p < 0.05$ as compared with LPS group.

were skeletally deformed, whereas there was no difference in fetal weight and crown–rump length (Table 4).

LPS-Induced Lipid Peroxidation and GSH Depletion

The effects of maternal LPS exposure on lipid peroxidation are presented in Figure 5. Maternal LPS exposure increased, to a lesser extent, TBARS content in maternal liver and placenta, whereas there was no difference in TBARS content in fetal tissue. The effects of maternal LPS exposure on GSH content in maternal liver, placenta, and fetal tissue are presented in Figure 6. Maternal LPS exposure significantly decreased the reduced GSH content in maternal liver and placenta. Interestingly, maternal LPS exposure also decreased the reduced GSH content in fetal tissue.

Nitric Oxide

To verify the effects of LPS on nitric oxide (NO) production, nitrite plus nitrate concentration in maternal serum or amniotic fluid was measured 6 h after the second LPS treatment. As shown in Figure 7, maternal LPS exposure significantly increased the level of nitrite plus nitrate in maternal serum or amniotic fluid.

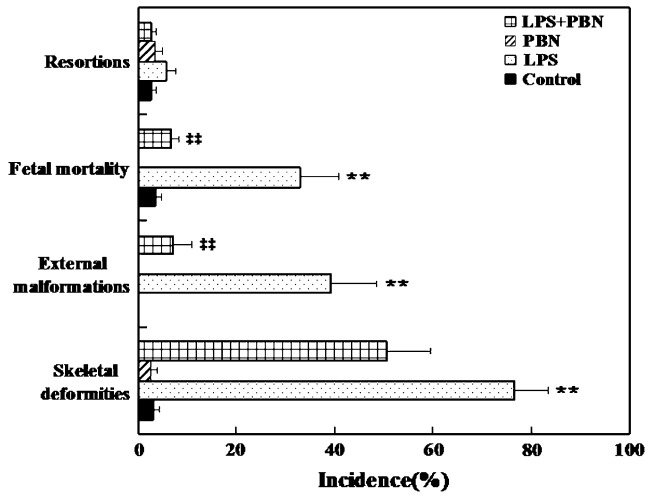

FIG. 4. The effects of PBN on LPS-induced fetal death and teratogenicity. The pregnant mice were intraperitoneally injected with two doses of LPS, one (20 μg/kg) injected on gd 8 and the other (20 μg/kg) injected 8 h later. The pregnant mice were sacrificed on gd 18. The numbers of live fetuses and dead fetuses were counted. Live fetuses were examined for external malformations and skeletal abnormalities. Fetal malformations were calculated per litter and then averaged per group. Data were expressed as means ± SEM. $^{**}p < 0.01$ as compared with control group. ‡‡$p < 0.01$ as compared with LPS group.

LPS-Induced Placental Nitrotyrosine Residues

In order to indirectly infer the production of peroxynitrite, nitrotyrosine residues in placenta were evaluated at 6 h after the second LPS administration. As shown in Figure 8, strong nitrotyrosine immunoreactivity was detected in placenta in mice treated with LPS.

The Protective Effects of PBN on LPS-Induced Fetal Death and Teratogenicity

To investigate the role of ROS on LPS-induced teratogenesis, the pregnant mice were pretreated with PBN (100 mg/kg, ip) 30 min before LPS administration. As expected, PBN pretreatment significantly attenuated LPS-induced lipid peroxidation in maternal liver and placenta (Fig. 5). Although PBN had no effect on LPS-induced GSH depletion in maternal liver and placenta, PBN pretreatment significantly attenuated LPS-induced GSH depletion in fetal tissue (Fig. 6). In addition, PBN pretreatment attenuated LPS-induced nitrotyrosine residues in mouse placenta (Fig. 8). The effects of PBN on LPS-induced fetal death and malformations are presented in Figure 4. PBN pretreatment greatly reduced LPS-induced fetal mortality (32.8 ± 7.9% vs. 6.4 ± 1.9%, $p < 0.01$) and the incidence of external malformations (39.1 ± 9.2% vs. 7.0 ± 3.8%, $p < 0.01$).

TABLE 4
Effects of a Two-Dose LPS Exposure on Fetal Weight and Crown–Rump Length

Groups	Litters (*n*)	Fetal weight (g, ±SEM)	Crown–rump length (mm, ±SEM)
Control	10	1.42 ± 0.06	24.1 ± 0.46
LPS	17	1.35 ± 0.06	23.4 ± 1.36
PBN	12	1.40 ± 0.05	24.0 ± 0.25
PBN + LPS	16	1.35 ± 0.03	23.6 ± 0.23

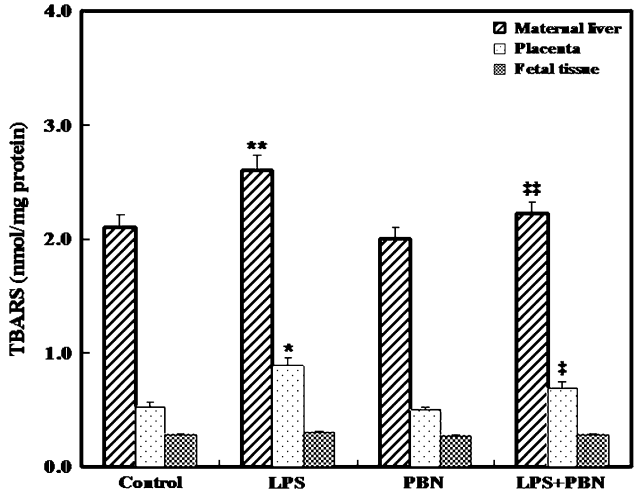

FIG. 5. Effects of PBN on LPS-induced lipid peroxidation. The pregnant mice were intraperitoneally injected with two doses of LPS, one (20 μg/kg) injected on gd 8 and the other (20 μg/kg) injected 8 h later. The pregnant mice were administered with PBN (100 mg/kg, ip) 30 min before LPS. The pregnant mice were sacrificed 6 h after the second LPS administration. TBARS contents were measured in maternal liver, placenta, and fetal tissue. Data were expressed as means ± SEM ($n = 12$) $^{**}p < 0.01$ as compared with control group. ‡$p < 0.05$, ‡‡$p < 0.01$ as compared with LPS group.

Although skeletal abnormalities were statistically indistinguishable between LPS-treated mice and LPS + PBN–treated mice, there was a trend for LPS + PBN–treated mice to have a lower incidence of skeletal abnormalities (Fig. 4).

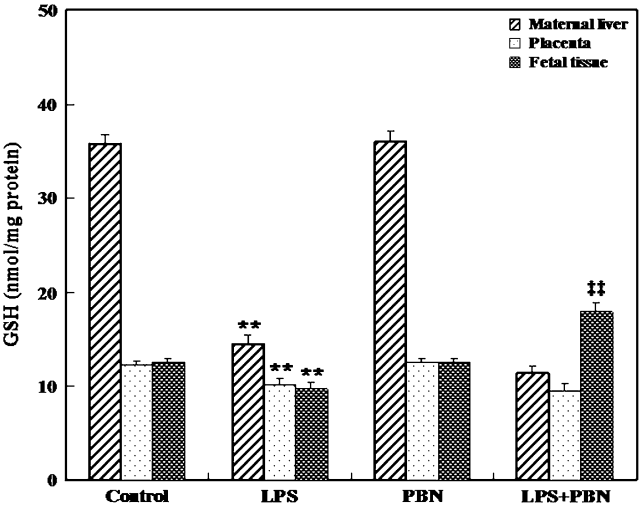

FIG. 6. Effects of PBN on LPS-induced GSH depletion. The pregnant mice were intraperitoneally injected with two doses of LPS, one (20 μg/kg) injected on gd 8 and the other (20 μg/kg) injected 8 h later. The pregnant mice were administered with PBN (100 mg/kg, ip) 30 min before LPS injection. The pregnant mice were sacrificed 6 h after the second LPS administration. GSH contents were measured in maternal liver, placenta, and fetal tissue. Data were expressed as means ± SEM ($n = 12$). $^{**}p < 0.01$ as compared with control group. ‡‡$p < 0.01$ as compared with LPS group.

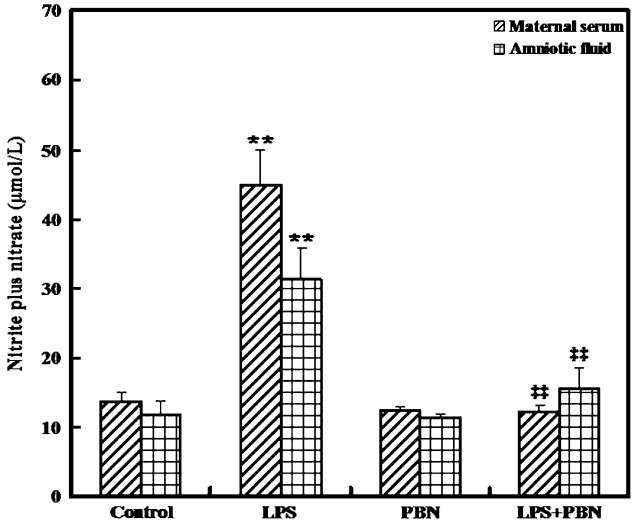

FIG. 7. Effects of PBN on LPS-induced nitric oxide production. The pregnant mice were intraperitoneally injected with two doses of LPS, one (20 μg/kg) injected on gd 8 and the other (20 μg/kg) injected 8 h later. The pregnant mice were administered with PBN (100 mg/kg, ip) 30 min before LPS injection. The pregnant mice were sacrificed 6 h after the second LPS treatment. Nitrite plus nitrate concentrations were measured in maternal serum and amniotic fluid. Data were expressed as means ± SEM ($n = 12$) $^{**}p < 0.01$ as compared with control group. ‡‡$p < 0.01$ as compared with LPS group.

DISCUSSION

The present study found that a 5-day LPS exposure resulted in pregnancy loss. In dams exposed to 30 μg/kg LPS, only 9 out of 17 dams completed the pregnancy. Importantly, a 5-day

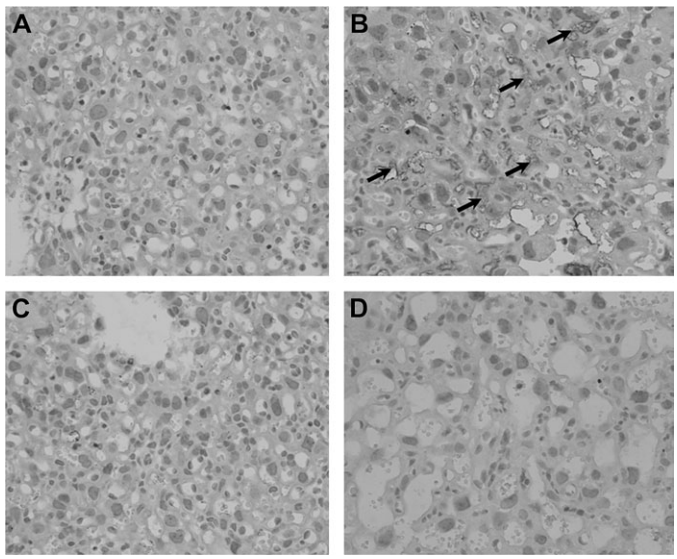

FIG. 8. LPS-induced nitrotyrosine residues in mouse placenta. There was only minimal nitrotyrosine immunoreactivity in placentas of mice treated with salt (A) or PBN (C) alone. Strong nitrotyrosine immunoreactivity (arrow) was observed in placentas of mice treated with LPS (B). LPS-induced nitrotyrosine residues were significantly attenuated by PBN pretreatment (D). Magnification: 200×.

LPS exposure significantly increased fetal mortality and the incidence of external malformations. The highest incidence of externally malformed fetuses was observed in fetuses from dams exposed to 20 μg/kg LPS, in which 34.9% of fetuses per litter were externally deformed. Recent studies showed that maternal exposure to a single dose LPS resulted in fetal death (Leazer *et al*., 2002; Xu *et al*., 2007). Therefore, it is very interesting whether a single dose of LPS also causes external malformations. Our preliminary study showed that less than 5% of fetuses per litter were externally malformed in dams exposed to a single dose LPS (either 20 or 40 μg/kg) during organogenesis (from gd 8 to gd 15). Interestingly, external malformations were observed in fetuses from dams exposed to only two doses of LPS on gd 8, in which 76.5% of litters and 39.1% of fetuses per litter were affected.

Several studies demonstrated that maternal LPS exposure enhanced placental expression of 4-hydroxy-2-nonenal–modified proteins, markers of oxidative stress (Ejima *et al*., 2000; Miller *et al*., 1996). A recent study found that perinatal LPS exposure upregulated the expression of heme oxygenase (HO)-1 in placenta (Zhang *et al*., 2007). The present study showed that maternal exposure to two doses of LPS on gd 8 resulted in lipid peroxidation and GSH depletion in maternal liver and placenta and increased NO production in maternal serum or amniotic fluid. Moreover, maternal LPS exposure initiated protein nitration, measured as a generalized strong nitrotyrosine immunoreactivity, in mouse placenta. It has been demonstrated that ROS are involved in LPS-induced fetal death, growth restriction, and preterm labor and delivery (Xu *et al*., 2006b). The present study investigated the role of ROS in LPS teratogenicity by pre-treating pregnant mice with the free radical spin-trapping agent PBN, which has been proven effective inhibiting the *in vivo* teratogenicity of the sedative drug thalidomide in rabbits (Parman *et al*., 1999) and the anticonvulsant drug phenytoin in mice (Parman *et al*., 1998). We found that PBN pretreatment significantly attenuated LPS-induced lipid peroxidation in maternal liver and placenta and alleviated NO production in maternal serum or amniotic fluid. In addition, PBN pretreatment attenuated LPS-induced nitrotyrosine residues in mouse placenta. Consistent with its antioxidant effect, PBN reduced LPS-induced fetal mortality. Importantly, PBN pretreatment abolished almost all external malformations, including exencephaly and encephalomeningocele, a hallmark of LPS teratogenicity. The considerable protection against LPS-induced teratogenicity and embryopathy provided by PBN pretreatment indicates that ROS may contribute, at least in part, to the teratologic mechanism.

An earlier study has demonstrated that LPS-induced developmental toxicity is a maternally mediated event (Leazer *et al*., 2002). Indeed, our recent study found that the increased level of tumor necrosis factor-alpha (TNF-α) in fetal liver and brain, which partially contributed to LPS-induced fetal death, might be transferred from either the maternal circulation or amniotic fluid (Ning *et al*., 2008; Xu *et al*., 2006a). The present study also showed that maternal LPS exposure resulted in lipid peroxidation and GSH depletion in maternal liver and placenta. However, a recent study demonstrated that maternal LPS exposure upregulated the expression of HO-1 in fetal liver, which was inhibited by radical trapping agent PBN (Li *et al*., in press). In the present study, we showed that maternal LPS exposure resulted in GSH depletion in fetal tissue, which was also alleviated by pretreatment with PBN. These data provides additional direct evidence for oxidative stress in fetal tissues. Indeed, ROS are known unstable molecules that generally cannot escape from the organ of formation, let alone travel from maternal to fetal tissue, without being scavenged by antioxidative molecules or enzymes. Thus, fetal GSH depletion probably requires that ROS be generated within the fetal tissue. The mechanism of ROS production in fetal tissue is unknown. Although maternally administered LPS could not pass through rat placenta to fetuses (Ashdown *et al*., 2006), TNF-α can transferred from maternal serum and amniotic fluid into fetuses (Ning *et al*., 2008). The *in vitro* study has demonstrated that TNF-α stimulates embryonic cells to produce ROS (Lin *et al*., 2004). Thus, ROS in the embryo tissues could be indirectly generated by TNF-α, transferred from either the maternal circulation or amniotic fluid.

As in the early phases of development embryonic antioxidant capacity is limited (Kobayashi *et al*., 2000), developing embryos are very sensitive to high levels of ROS, especially during early organogenesis. Although the mechanism for ROS-mediated teratogenesis remains unclear, the teratogenicity of several xenobiotics is thought to depend at least partially upon their bioactivation to electrophilic and/or free radical reactive intermediates that covalently bind to or oxidize cellular macromolecules such as DNA, protein, and lipid, resulting in intrauterine fetal death or teratogenesis (Ornoy, 2007; Wells *et al*., 1997). For example, thalidomide is bioactivated in the fetus by prostaglandin H synthase to a metabolite that can oxidize DNA and GSH, as it generates ROS. That is apparently a major mechanism for teratogenesis of thalidomide was proven by the fact that in pregnant rabbits, teratogenicity was reduced by pretreatment with free radical spin-trapping agent PBN, as DNA oxidation was markedly reduced. In mice, that are very resistant to the teratogenic action of thalidomide, DNA oxidation was not enhanced even by high doses of thalidomide (Ohkawa *et al*., 1979).

The protection of PBN against LPS-induced teratogenicity and embryopathy may have therapeutic implications. PBN protects against teratogenicity of drugs, such as phenytoin and thalidomide, which are known to induce embryonic and fetal oxidative stress. In a recent study, Wentzel *et al.* (2006) administered to rats 20% ethanol to the drinking water throughout pregnancy, and studied the effects of the addition of 5% vitamin E to the food, on the outcome of ethanol-exposed pregnancies. The addition of the vitamin E markedly alleviated ethanol-induced fetal anomalies and death. In addition, melatonin, NAC, PBN, and ascorbic acid have been

shown to protect against LPS-induced intrauterine fetal death, intrauterine growth restriction, and preterm labor and delivery (Chen *et al*., 2006a, b; Xu *et al*., 2005, 2006b). Therefore, antioxidants may be used as potential embryoprotective agents for clinical therapy in high-risk situations in which pregnant women are infected with bacteria, or situations in which pregnant women are exposed to ROS-initiating chemicals.

In summary, the present results allow us to reach the following conclusions. First, maternal LPS exposure during organogenesis can result in external and skeletal abnormalities. Importantly, external and skeletal malformations were also observed in fetuses from dams exposed to only two doses of LPS on gd 8, suggesting that short-term LPS exposure is teratogenic. Second, ROS contribute, at least partially, to LPS-induced teratogenesis. Thus, antioxidants may have a potential preventive and therapeutic utilities for protecting against LPS-induced teratogenicity.

SUPPLEMENTARY DATA

The color version of figures 1, 3, and 8 are available as supplementary data online at http://toxsci.oxfordjournals.org/.

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